Human Tooth Developmental Anomaly: Odontoblastic Differentiation in Hamartomatous Calcifying Hyperplastic Dental Follicles Presenting DSP, Nestin, and HES1

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Article

Human Tooth Developmental Anomaly: Odontoblastic Differentiation in Hamartomatous Calcifying Hyperplastic Dental Follicles Presenting DSP, Nestin, and HES1

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Abstract: Hyperplastic dental follicles (HDFs) represent odontogenic hamartomatous lesions originating from pericoronal tissues and are often associated with impacted or embedded teeth. These lesions may occasionally feature unique calcifying bodies, known as calcifying whorled nodules (CWN), characterized by stromal cells arranged in a whorled or spiral fashion. CWNs are typically observed in multiple calcifying hyperplastic dental follicles or regional odontodysplasia. In our study, we examined 40 cases of HDFs, including nine instances with characteristics of CWNs, which were infrequently accompanied by odontodysplasia. Histological examination was conducted on all 40 cases, with immunohistochemical analysis performed on 21 of them. Among the cases with CWN, nine affected a single embedded tooth, with one exception. CWNs exhibited diverse calcifications featuring sparse or entirely deposited psammoma bodies, and some displayed dentinoid formation. Immunohistochemically, stromal cells of HDFs were frequently positive for CD56 and nestin. In contrast, CWNs were negative for CD56 but positive for nestin and HES1, with a few DSP-positive calcified bodies. Our results revealed that hamartomatous calcifying HFDs can impact multiple and single-embedded teeth. CWNs composed of nestin and HES1-positive ectomesenchymal cells demonstrated the potential to differentiate into odontoblasts and contribute to dentin matrix formation under the influence of HES1. This study is the first report documenting odontoblastic differentiation in HDFs.

Keywords: odontoblastic differentiation; hyperplastic dental follicle; calcifying whorled nodule; nestin; dentin sialoprotein; hairy and enhancer split 1

1. Introduction

Embedded or impacted teeth represent developmental anomalies that can lead to various diseases, including odontogenic cysts and tumors [1]. Hyperplastic dental follicles (HDFs) are uncommon pathological conditions associated with embedded teeth, occasionally posing a histological challenge due to their similarity to central odontogenic fibromas [2]. This lesion exhibits diverse histological features, encompassing myxofibromatous tissue, with or without various types of calcified tissue, and features reminiscent of odontogenic epithelial tumors [3–5]. The majority of these lesions demonstrate myxofibrous hyperplasia or a combination of odontogenic epithelial proliferation resembling ameloblastoma [5], squamous odontogenic tumors, and various calcifications, including psammoma bodies [4].

Multiple calcifying hyperplastic dental follicles (MCHDF) represent a rare pathological condition initially described by Sandler et al. in 1988 [6]. The term MCHDF was coined by Gardner DG and Radden B in 1995 [7], and subsequently, numerous authors reported similar cases using this nomenclature [8–14]. These cases share common histological features, displaying small droplets of calcified material with a whorled pattern and varying amounts of odontogenic epithelial islands

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within fibrous or myxofibrous tissue. Furthermore, some instances are accompanied by regional odontodysplasia [7]. This type of calcification may be found in solitary hyperplastic dental follicles, occasionally forming conglomerates of enameloid, cementoid, and psammomatous calcifications. The initiation of tooth formation involves two integral components: the surface epithelium and the underlying mesenchyme, both originating from cranial neural crest cells [15]. Notably, human dental pulp stem cells exhibit positivity for CD24, CD117, and CD146, while lacking nestin expression [16,17]. In contrast, dental follicle spindle cells, characterized by a notable population of neural stem/progenitor cells expressing nestin [18], demonstrate the capability to differentiate into neural cells [19]. Both immature and mature odontoblasts, derived from neural crest cells, express nestin, dentin sialoprotein (DSP) protein, and mRNA [20,21].

This study focused on the examination of the structure of small droplets of calcified material exhibiting a distinctive whorled or spiral pattern, termed calcifying whorled nodules (CWN). While CWN is a characteristic feature of MCHDF, it is also identified in isolated cases of calcifying hyperplastic dental follicles (CHDF). The hypothesis posits that solitary or multiple CHDF instances may constitute hamartomatous overgrowths of neural crest cells that incompletely differentiate into odontoblasts, resulting in the formation of dentinoids or calcified particles. To elucidate the nature of CWNs, we conducted immunohistochemical investigations on dental follicles, including CHDFs, carefully selected from our archival specimens. This presentation provides compelling immunohistochemical profiles of CWNs, shedding light on potential odontoblastic differentiation within dental follicles.

2. Materials and Methods

2.1. Sample characteristics

Formalin-fixed, ethylenediaminetetraacetic acid-treated, and paraffin-embedded (FFPE) human samples were sourced from the archives of the Department of Surgical Pathology at Matsumoto Dental University Hospital. The inclusion criteria were as follows: 1) radiolucent lesions around embedded tooth/teeth; 2) fibrous or myxoid stroma with or without small dentinoids; and 3) markedly thickened cyst walls composed of significant myxoid stroma or dentinoid hard tissue in odontogenic cysts such as dentigerous cysts or odontogenic keratocysts caused by hyperplastic dental follicles. Patients with odontomas and dominant hard tissue formation composed of dentinoid and enameloid tissues were excluded. In total, 40 patients were selected, with the anterior incisors, premolars, and molars accounting for 30%, 5%, and 65% of the patients, respectively.

Third molars with incomplete root formation, extracted under the diagnosis of unerupted teeth, served as positive control tissues for immunohistochemical analyses. The study adhered to the principles of the 2008 Declaration of Helsinki, and the study protocol received approval from the Ethics Committee of Matsumoto Dental University (approval number 0322).

2.2. Immunohistochemistry

Histological sections, three micrometers thick, were prepared from FFPE samples. Following deparaffinization and hydration, optimized antigen retrieval methods were employed, as outlined in Table 1 (dentin sialoprotein (DSP): proteinase K for 3 min; CD56/NCAM and CD117/c-kit: heat-induced epitope retrieval (HIER) in a pressure cooker with antigen retrieval solution (pH 6.0) (#715281, Nichirei Bioscience, Tokyo, Japan); hairy and enhancer split 1 (HES1) and nestin: HIER in a pressure cooker with antigen retrieval solution (pH 9.0) (#415201, Nichirei Bioscience)). Endogenous peroxidase activity was blocked by incubation in a 3% H2O2 aqueous solution for 15 min, and nonspecific reactions were blocked by incubation in a protein block solution (#X090930-2, Agilent Technologies, Santa Clara, CA, USA) for 10 min. After incubation with primary antibodies for 1 h at 24 °C, the sections were washed three times with phosphate-buffered saline and incubated with a horseradish peroxidase-conjugated secondary antibody (#424152, Histofine Simple Stain MAX PO Multi, Nichirei Bioscience) for 30 min. For visualization, sections were incubated with 3-3'-

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diaminobenzidine tetrahydrochloride (DAB) (#K3468, Agilent Technologies), then counterstained with hematoxylin.

Third molar sections displaying incomplete root formation were employed as positive controls for the immunohistochemical analyses. Negative control sections were meticulously prepared using the procedure, except for omitting the primary antibodies. This approach aimed to discern and identify any nonspecific reactions.

2.3. Statistical analysis

The age distributions of patients, both with and without CWN were subjected to analysis through the Mann–Whitney U test. Examination of the locations of CHDFs, nestin, HES1, and DSP between CHDFs and other HDFs, as well as the comparison of HES1-immunoreactions between DSP-positive and negative cases, was conducted utilizing Fisher's exact test. Post-hoc analysis was performed using Holm's test. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [22]. EZR is a user-friendly graphical interface for Rwww.r-project.org (R Foundation for Statistical Computing, Vienna, Austria). *P-values <0.05* were considered statistically significant.

Table 1. Panel of primary antibodies.

Antibody	Product number	Source	Clone	Treatment	Dilution
CD56/NCAM	M730429-2	Agilent	123C3	HIER	1:100
CD117/c-kit	A450229-2	Agilent	polyclonal	HIER	1:50
DSP	sc-73632	Santa Cruz Biotechnology	polyclonal	Digestion	1:4000
HES1	#11988	Cell Signalling Technology	D6P2U	HIER*	1:200
Nestin	HPA007007	Sigma-Aldrich	polyclonal	HIER*	1:5000

DSP: dentin sialoprotein; HES1: hairy and enhancer split 1; HIER: heat induced epitope retrieval (pH6.0), *(pH9.0).

3. Results

3.1. Clinical characteristics of (CHDFs

In this study, we examined 40 cases of HDFs to explore the clinical characteristics of CHDFs. Notably, nine cases (22%) exhibited CWNs, a distinctive feature of MCHDFs. Only two cases (5%) displayed odontodysplasia, accompanied by MCHDF and solitary CHDF. Statistical analysis revealed no significant difference (p=0.228) in the median ages of all HDFs and CHDFs, which were 16 and 15 years, respectively. Sex predilection was not observed. The molar region was the most frequently affected by both HDFs (67.7%) and CHDFs (55.6%), followed by the anterior teeth (29%, 33.3%) and premolars (3.2%, 11.1%), with no statistically significant difference (p=0.426). CHDFs predominantly developed in the lower third molars (44.4%), followed by the upper anterior teeth (33.3%) (Figure 1).

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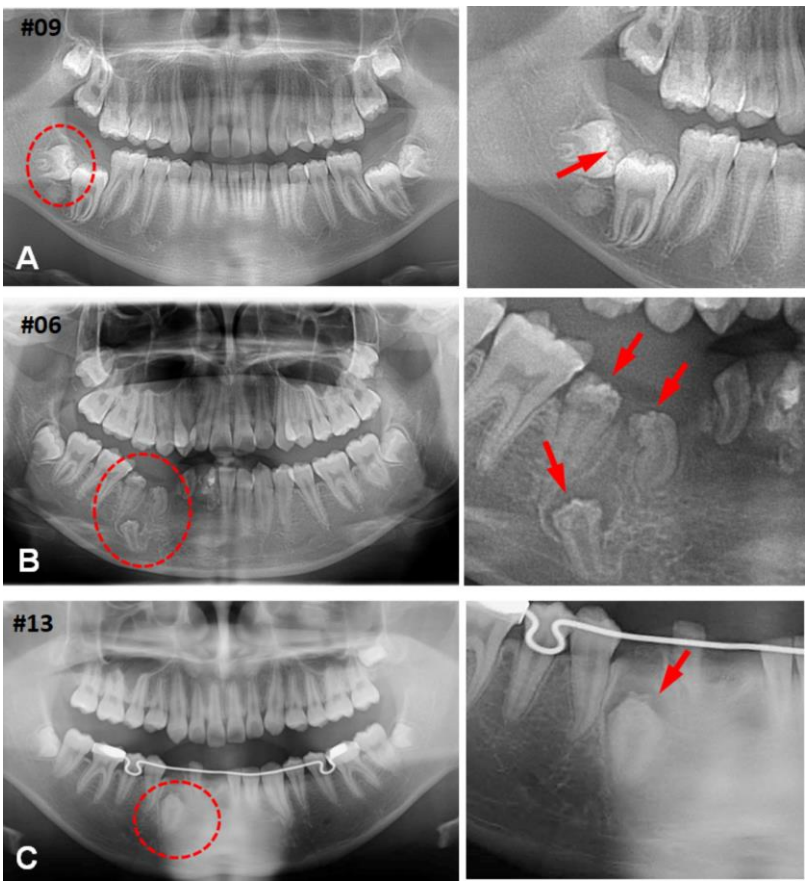

Figure 1. Representative dental panoramic radiographic images and enlarged pictures of dotted circles: a hyperplastic dental follicle (arrow) in case 6 affecting the impacted mandibular third molar (A); multiple calcifying hyperplastic dental follicles (arrows) in case 9 (B); solitary hyperplastic dental follicle (arrow) in case 13.

3.2. Histological changes of HDFs

The fibrous lesions that enveloped the crowns of the impacted teeth exhibited varying degrees of thickening, often displaying fibrous changes in 79% of cases and myxoid changes in 67%. Giant cells were detected in only one instance, comprising 3% of the cases. Most cases (95%) featured diverse numbers of epithelial islands. In 36% of cases, the reduced enamel epithelium exhibited reticular or massive proliferation, occasionally accompanied by ghost cells (18%) or the deposition of enamloid matrices (5%). Mesenchymal calcification was observed in approximately half of the cases, categorized into three types: psammomatoid calcification (49%), calcifying whorled nodules (23%), and dentinoid formation (41%) (Figure 2).

Calcifying whorled nodules exhibited diverse forms of calcifications. Certain nodules consisted of spindle cells gathering in a spiral configuration with minimal calcification. Other nodules demonstrated heightened deposition of small calcifications. On occasion, a diminutive whorled nodule contained psammoma bodies, and certain nodules underwent dentinoid formation around the psammoma bodies (Figure 3).

3.2. Immunohistochemical profiles of CWNs in CHDFs

Immunohistochemically, the stromal spindle cells of CHDFs exhibited positive CD56 expression in most cases (81.0%), while the spindle cells of CWNs were consistently negative. Focal CD56-positive epithelial islands were identified in 66.7% of cases. HES1 demonstrated rare positivity in stromal spindle cells (6.7%), contrasting with the positive expression observed in 66.7% of spindle cells in CWNs and 56.3% of odontogenic epithelium cases. Nestin displayed positive expression in 95% of stromal spindle cells and 100% of CWNs, yet it was completely negative in the odontogenic

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epithelium. The central areas of concentric calcified materials in CWNs were only focally positive for DSP in 50% of cases. Notably, all DSP-positive cases exhibited positivity for nestin, and 75% of DSP-positive cases were positive for HES1. Ultimately, this study elucidated a propensity for positive reactions to nestin, HES1, and DSP in the spindle cells of CWNs. CD117 exhibited complete negativity in all cases (Figure 4 and Table 2). The immunoreactivity of stromal spindle cells against CD56 and nestin demonstrated no significant difference between CHDFs and non-CHDF/HDFs without CWNs (CD56, *p=0.618;* nestin, *p=1*).

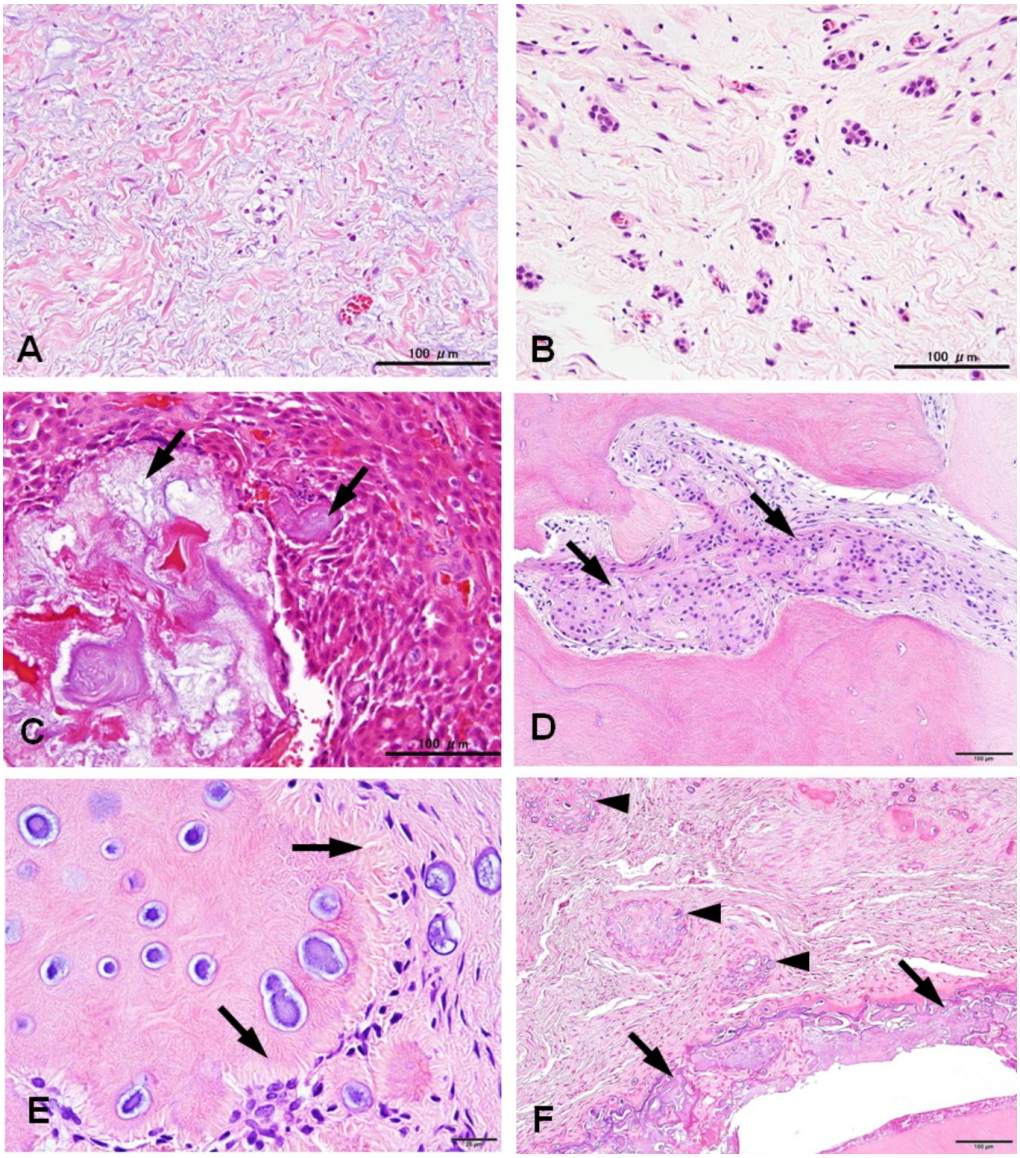

Figure 2. Histological variations of hyperplastic dental follicles: the stroma of the lesion is frequently myxo-fibrous (A), accompanied by the proliferation of small islands of the odontogenic epithelium (B); rarely, enamloid materials are present in the proliferated epithelium (C); dentinoid formation frequently accompanies juxtaposed odontogenic epithelium (arrows) (D); some cases show dentinoid nodules with a structure reminiscent of dentinal tubules (arrows) and psammomatous calcification surrounded by spindle cells forming a whorled structure, namely a calcifying whorled nodule (E); a representative case 13 shows many calcifying whorled nodules (arrowheads) distributing around a tooth crown with odontodysplasia (arrows) (F). All bars represent 100 μm except for E (20 μm).

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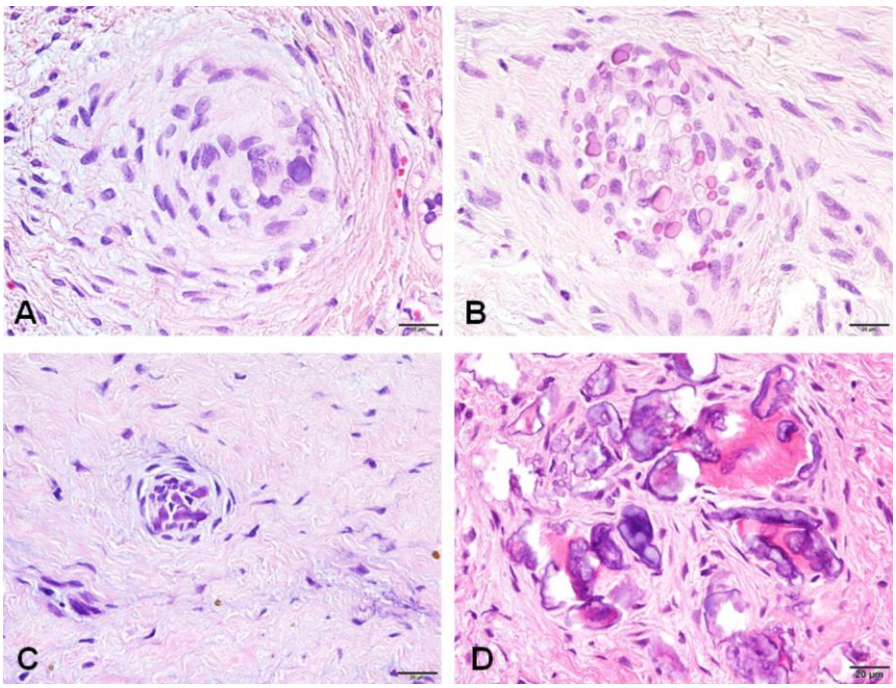

Figure 3. Histological variations of calcifying whorled nodules: spindle cells aggregate in a whorled or spiral fashion with scant calcification (A); some nodules show an increased deposition of small calcification (B); occasionally, a small whorled nodule is occupied by psammoma bodies (C); some nodules develop dentinoid formation around psammoma bodies (D). All bars represent 20 μm.

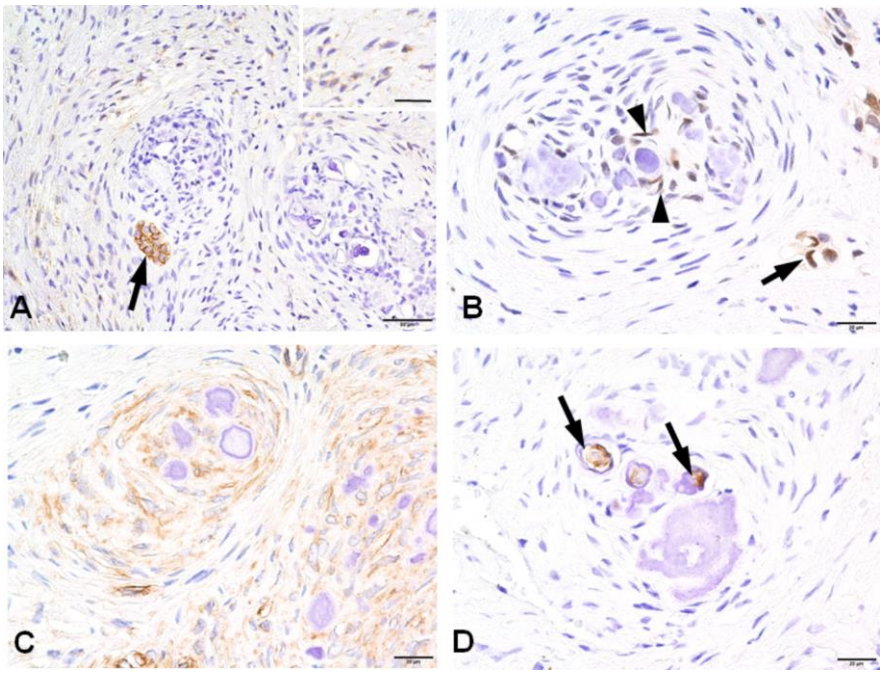

Figure 4. Immunohistochemical findings of calcifying whorled nodules: Stromal spindle cells are positive for CD56 (inset), but calcifying whorled nodules are negative. There is a CD56-positive epithelial island (arrow) (A); a few spindle cells (arrowheads) in a calcifying whorled nodule and some epithelial cells (arrows) are nuclear-positive for hairy and enhancer of split-1 (HES1) (B); spindle cells aggregating in a wheel fashion are entirely positive for nestin (C); the central areas of concentric calcified materials are focally positive for dentin sialoprotein (DSP). All bars represent 20 μm except for A (50 μm).

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Table 2. Summary of immunophenotypes of components of hyperplastic dental follicles.

Components	CD56	Nestin	HES1	CD117	DSP
Stroma	+	+	+	-	-
Epithelial islands	+	-	+	-	-
CWNs	-	+	+	-	+
Psammoma bodies	-	-	-	-	-
Dentinoid	-	-	-	-	-

CWNs: calcifying whorled nodules; HES1: hairy and enhancer split 1; DSP: dentin sialoprotein.

4. Discussion

Aberrant psammomatous calcification may manifest in syndromic or regional hypoplasia of teeth, albeit infrequently impacting dentition. Regional odontodysplasia, initially documented by Hitchin in 1934, exhibits cementum-like and psammomatous calcification, enveloped by a whorled fibrous stroma referred to as CWN in this investigation [23]. Multiple calcifying hyperplastic dental follicles represent a rare condition characterized by numerous unerupted teeth with substantial calcifications and remnants of odontogenic epithelium within their enlarged dental follicles, often accompanied by odontodysplasia [6–11,14]. Histologically, all cases demonstrated similarities, showcasing calcified materials with a whorled or spiral structure akin to CWN. HDFs are odontogenic hamartomatous lesions arising from pericoronal tissues associated with impacted or embedded teeth [24], presenting diverse histological findings, as previously documented [4]. While CWNs are traditionally considered a distinctive feature of MCHDFs, this study reveals that CWNs may also manifest in solitary embedded teeth exhibiting hyperplastic changes, denoted as solitary CHDFs.

Notably, this study breaks new ground by providing immunohistochemical insights into the stromal cells of CWNs, a facet not previously explored. The immunohistochemical profile of CWNs appears to align with that of odontoblasts during the latter stages of differentiation.

The dental follicles of impacted third molars infrequently contain immature fibroblasts exhibiting phenotypic features of stromal stem cells expressing CD44 and CD56, constituting less than 2% [25]. In contrast, CD56-positive cells were identified in 81% of cases within our series. This observation underscores the prevalent presence of stromal stem cells in HDFs, suggesting their capacity for differentiation into odontoblasts. Notably, HDFs diverge from dental pulp stem cells, as the latter express CD117/c-kit [17], a marker absent in all HDF cases analyzed. This finding elucidates the absence of odontogenic components associated with odontomas in HDFs. Morphological examinations further support this conclusion, as no calcified tissue consisting of dentinoid and enameled tissues was observed in HDFs. This precludes the possibility of minute odontomas originating from impacted teeth.

To comprehend tooth initiation, it is imperative to consider two fundamental components: the surface epithelium and the underlying mesenchyme. The dental mesenchyme, crucial for tooth development, originates from cranial neural crest cells migrating into the frontonasal process and the first branchial arch [15]. Nestin, initially characterized in neural stem cells, is a cytoskeletal intermediate filament and serves as a neuroepithelial stem cell marker. Despite its association with neural cells, recent evidence from in vivo models and humans highlights the presence of nestin-positive cells with progenitor and/or regulatory functions in various tissues [26]. Notably, odontogenic ectomesenchymal cells, akin to neural cells in origin [15], exhibit convincing evidence of nestin. The dental follicle, an ectomesenchymal tissue enveloping the developing tooth germ, is a noteworthy example. Human follicle cells, distinctively, demonstrate the ability to differentiate into neural cells. In cultured spindling dental follicle cells, the expression of neural markers such as nestin, β-III-tubulin, and S100β is evident. Additionally, there is an up-regulation of Musashi-1 and Musashi-2, MAP2, GFAP, MBP, and SOX10 [19].

Nestin serves as a prominent dentin follicle marker [27] and is notably expressed in differentiated odontoblasts [21]. While there is limited documentation on nestin expression in odontogenic neoplasms or related lesions, it has been observed that all ameloblastomas and

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malignant ameloblastomas exhibit negativity for nestin. However, in odontogenic mixed tumors, nestin immunoreactivity is evident in the odontogenic ectomesenchyme, particularly in regions adjacent to the odontogenic epithelium. Moreover, positive nestin expression is observed in odontoblasts, pulp cells, and cells adhering to dentin in odontomas [28]. Nestin has also been utilized as a differentiation marker for odontoblasts during tooth development. In the later stages of odontogenesis, the unmineralized matrix gradually transforms into mature dentin. In this context, DSP and nestin are recognized as markers of late-stage odontoblastic differentiation [29]. Notably, experimental evidence indicates the co-expression of nestin and DSP during the late stage of odontoblast differentiation, encompassing both pre-odontoblasts and mature odontoblasts [20]. The DSP domain plays a crucial role in facilitating mesenchymal cell differentiation and mineralization [30]. Calcifying whorled structures exhibit positive reactions to nestin and DSP, indicative of the late stage of odontoblast differentiation. Morphologically, some calcifying whorled structures contain dentinoid materials with tubule-like dentinal structures. Considering both the immunohistochemical findings and the morphological features of dentinoid formation, it is conceivable that certain constituent cells of dental follicles possess the potential to undergo differentiation into odontoblasts. This report represents the initial documentation of odontoblastic differentiation in HDFs.

The Notch signaling pathway holds a paramount status as an ancient and remarkably preserved mechanism. Its involvement extends across diverse physiological processes, including cell differentiation and regeneration, while also playing a pivotal role in pathological conditions such as carcinogenesis. Specifically, the signaling of Notch operates in a manner that negatively regulates the differentiation of neural stem cells, directing them away from the neuronal lineage [31]. Genes featuring suppressors of hairless paired sites (SPS) or Notch transcription complexes at DNA-binding sites are inclined to be high-affinity targets for Notch. Consequently, Hes1, a prototypical SPS-containing Notch target, exhibits responsiveness to Notch1 [32]. The upregulation of Hes1 may be attributed to an increased activity within the Notch signaling pathway, potentially contributing to the proliferation of tumors [33]. Conversely, Hes1 acts as an inhibitor of Notch signaling, thereby influencing the differentiation of embryonic stem cells by suppressing Notch signaling via Hes1 [34]. The expression of Hes1 has been identified in the stellate reticulum cells and the outer dental mesenchyme [35]. In dental pulp subjected to Ca(OH)2 treatment, there was an observed upregulation of Hes1, accompanied by increased expression of Notch1, Notch2, and Notch3. Conversely, diminished Notch/Hes1 signaling has been linked to a reduction in tooth size [36]. These investigations indicate that Hes1 plays a significant role in odontogenesis. Furthermore, the suppression of Notch signaling has been found to facilitate odontoblastic differentiation in HES1-positive CWN cells.

In vivo, hyperplastic dental follicles potentially serve as valuable models for understanding odontogenesis in human ectomesenchymal cells. Unfortunately, the scope of this study was limited by the availability of specimens suitable for immunohistochemical examination, and none were deemed appropriate for molecular analysis. Consequently, our findings indicate a tendency for the immunophenotypes of constituent cells within CWNs to express nestin, DSP, and HES1. However, it is imperative to underscore that additional investigations are imperative to substantiate and validate these preliminary results.

5. Conclusions

Hamartomatous calcifying hyperplastic dental follicles not only impact multiple teeth but also manifest in single embedded teeth. Remarkably, characteristic features of MCHDs, such as CWNs, may also be present in solitary HDFs without concurrent odontodysplasia. The transition of CD56-positive to CD56-negative status in ectomesenchymal cells of HDFs is noteworthy. Subsequently, mesenchymal cells forming whorled nodules may harbor the potential to differentiate into odontoblasts, thereby producing a dentin matrix under the influential guidance of HES1.

Supplementary Materials: The following supporting information can be downloaded at: https://zenodo.org/records/10253921; Table S1: Clinical summary of HDFs; Table S2: Histological findings of HDFs; Table S3: Immunohistochemical findings of HDFs.

Author Contributions: Conceptualization, H.H.; methodology, HH, K.S and T.O.; formal analysis, H.H; writing—original draft preparation, H.H.; writing—review and editing, H.H, K.S and Y.O.; supervision, Y.O. All authors have read and agreed to the published version of the manuscript.".

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Institutional Review Board Statement: The study protocol was approved by the Ethics Committee of Matsumoto Dental University (approval number 0322).

Informed Consent Statement: Informed consent was obtained using an opt-out method because all samples had been embedded in paraffin and stored in departmental archives.

Data Availability Statement: All data were included in this manuscript and tree supplemental Tables.

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Conflicts of Interest: The authors declare no conflict of interest.

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